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High hydrostatic pressure activates gene expression through Msn2/4 stress transcription factors which are involved in the acquired tolerance by mild pressure precondition in *Saccharomyces cerevisiae*

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Abstract **Msn2 and Msn4 transcription factors activate expression of stress-responsive element (STRE) controlled genes in response to various stresses triggering the environmental stress response in *Saccharomyces cerevisiae*. Although high hydrostatic pressure is known to induce gene expression modification in yeast, the transcription factors involved in this response are currently uncharacterized. In this work, we show that elevated pressure activates STRE dependent transcription through Msn2/4, which are also required for cell resistance and cell adaptation to high pressure. Moreover, it was demonstrated that *HSP12* induction after a 50 MPa treatment is largely dependent on Msn2/4, while other transcription factors are involved in *HSP12* over-expression after a 100 MPa treatment.**

Keywords: Stress; Hydrostatic pressure; Msn2; Msn4; cAMP

1. Introduction

Organisms subjected to high pressure are compelled to withstand structural alterations in their biomolecules and cellular organelles towards a more compact form. Among other effects, membrane fluidity is reduced by increasing the packing of the lipids, and protein conformation is disturbed, mainly by the insertion of water molecules in hydrophobic cavities [1]. Although the physiological effects of hydrostatic pressure in *Sacchromyces cerevisiae* are still poorly understood, it has been shown that pressures around 50 MPa cause intracellular acidification and impair tryptophan transport across the membrane [2].

To understand better how yeast cells deal with pressure stress, microarray analysis of *S. cerevisiae* subjected to different ranges of hydrostatic pressure has been performed by two groups [3–5]. One assay was carried out after a 16 h growth at 30 MPa, a treatment that causes growth delay, but not cell death. In these conditions, the highest up-regulated genes were involved in lipid synthesis and amino-acid metabolism [4]. Exploring a range of pressure able to reduce cell viability, Fernandes et al. [5] conducted a microarray analysis of gene expression after a treatment of 200 MPa for 30 min, which revealed a typical stress response profile. The majority of the up-regulated genes are involved in stress defense and carbohydrate metabolism, while most of the repressed ones are involved in cell cycle progression and protein synthesis. The other results come from a transcriptome analysis carried out after a treatment of 180 MPa at 4 °C [3]. Despite the difference in the experimental designs, some common features can be seen in the last two arrays, for example the induction of small heat shock proteins (HSPs) such as *HSP12, HSP26, HSP30* and genes involved in protein degradation, such as *UBI4*.

Other pieces of evidence indicating that pressure can elicit a stress response come from cross protection studies. A non-lethal pre-treatment of 50 MPa for 30 min, has enhanced survival of *S. cerevisiae* cells against severe treatments such as heat, ultra-cold shock and high-pressure [6]. Moreover, pressure response seems to have some features in common with other stresses such as heat, oxidative stress, ethanol and cold shock, as a pretreatment with these conditions also induces pressure resistance [7,8].

Therefore, in the present work, we explore the involvement of the so-called "general stress" transcription factors Msn2 and Msn4 in the regulation of gene expression induced by hydrostatic pressure. These two homologous zinc finger proteins bind to the pentanucleotide C_4T, the *cis*-acting stress-responsive element (STRE) located at the promoter region of a large number of stress-responsive genes [9,10]. Msn2/4 activation is controlled at multiple levels. Under physico-chemical stresses or nutritional depletion, they migrate to the nucleus [11] and display an oscillatory behavior shuttling between the nucleus and cytoplasm [12], furthermore, the binding of Msn2 to DNA is controlled by stress and, possibly, by protein kinase Gsk3 [11,13]. RAS-cAMP pathway negatively regulates Msn2/4 translocation to the nucleus preventing transcription of STRE regulated genes [11]. The transcriptional activity of Msn2 is itself activated by stress,

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Abbreviations: HP, high pressure; CFU, colonies forming unit; STRE, stress-responsive element; ESR, environmental stress response; HSP, heat shock protein; LDPE, low density poly-ethylene; RCN, relative copy number; PT, pressure treated

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although the exact molecular mechanism is still unknown [14].

Among the genes up regulated by a pressure treatment of 200 MPa for 30 min [5], 40% have one or more STRE motif in their promoter region, and about 30% have already been reported to be at least partially dependent on Msn2/4 under oxidative, acid or heat stress [15,16]. Moreover, micro arrays performed after other high pressure conditions [3,4] also indicated up regulation of known Msn2/4 controlled genes, thus suggesting Msn2 and Msn4 activation by high pressure conditions.

Genome wide analysis has shown that most of the genes controlled by Msn2/4 belong to a group of genes that are stereotypically altered following stressful environmental transitions, defined as the environmental stress response (ESR) [15,16]. ESR represents a common response elicited by different stresses, protecting critical cell functions during periods of stress and conferring cross protection against different stress conditions [15]. However, the ultimate stress response is known to be unique to each stress condition, with other factors acting besides Msn2/4, for example, Hsf1, Yap1 or Hog1 affecting gene expression in response to heat shock, oxidative and osmotic shock, respectively [17].

In this work, we investigate if high hydrostatic pressure stress can elicit the activation of Msn2/4 transcription factors and if the response mediated by those factors is important to yeast cell survival under pressure conditions.

2. Materials and methods

2.1. Yeast strains

The following strains were used: Wmsn2msn4 (MATa *ade2 his3 leu2 trp1 ura3 msn2-D3::HIS3 msn4::TRP1*) and the wild-type strain W303-1A (MATa *ade2 his3 leu2 trp1 ura3*) [18]; BYhog1 (MATa *his3 leu2 met15 ura3 hog1::KANMX4*); BYyap1 (MATa *his3 leu2 met15 ura3 yap1::KANMX4*); (kindly provided by Dr. Claudio A. Masuda, IBqM, UFRJ) and the isogenic strain BY4741 (MATa *his3 leu2 met15 ura3*). β-Galactosidase assays were performed with OL556-STRE (MATa/MATα cdc25-5/cdc25-5 his3/his3 leu2/leu2 trp1/TRP1 rca1/rca1 ura3/ura3 STRE-LacZ::URA) [19] and also with W303-1A-STRE and Wmsn2msn4-STRE which contain the reporter gene *lacZ* under the control of four STRE motifs integrated into *URA3* locus.

2.2. Growth condition and hydrostatic pressure treatment

Yeast strains were grown overnight in YPD (2% glucose, 1% yeast extract, 2% peptone) at 27 °C to exponential growth phase (OD_{600} 1.0). When added to the medium, cAMP (Sigma) was 3 mM. Pressure equipment was the same as previously described [20]. Briefly, yeast samples were set in low density poly-ethylene tubes (LDPE) tightly stoppered and immersed in a stainless steel chamber connected to a manual piston screw pump and gauge. Ethanol was pumped into the chamber until the final pressure was reached. Yeast cells were subjected to hydrostatic pressure ranging from 30 MPa to 200 MPa for 30 min or 1 h at 37 °C or 27 °C. The time to attain the maximum pressure and to release it was less than 1 min. The control samples were set in the LDPE tubes used for the pressure treatment and held at similar conditions, except for pressure or temperature. For preconditioning experiments, cells were treated with 50 MPa for 30 min or 1 h and after 15 min of incubation at 1 atm, the culture was subjected to 200 MPa for 30 min.

2.3. Cell viability

Cells were plated on solidified YPD medium after appropriate dilution. Viability was determined by counting the colony-forming units (CFU) 48 h after the treatment. Survival rate was compared to the control sample. All plates were made in duplicate for each treatment.

2.4. RNA isolation, semi-quantitative RT-PCR and Real-time PCR

Total RNA was extracted and first strand synthesis of cDNA and semi-quantitative RT-PCR was performed as previously described [5]. Quantitative Real Time PCR was conducted using Abi Prism 7500 Sequence Detection System (Applied Biosystems) and analyzed by SDSv1.2.3 software (Applied Biosystems). Amplification of *HSP12* and *ACT1* was performed in 25 μl reaction volume containing 2.5 μl of SYBRGreen PCR Master Mix (Applied Biosystems), 1 μl of 1:40 diluted cDNA and 200 nM of each specific primer. PCR parameters were the same as described for conventional RT PCR [5]. The melting-curve analysis showed the specificity of the amplifications. Relative expression was calculated as in [21]. Threshold cycle (Ct), which inversely correlates with the target mRNA level, was measured as the cycle number at which the reporter fluorescent emission appeared above the background threshold. Briefly, the mean Ct for three replicates of *HSP12* amplicon was subtracted from the mean Ct for three replicates of the reference ACT1 mRNA in each sample to obtain ΔCt, [ΔCt = Ct (gene) − Ct (actin)]. Relative copy number (RCN) in the pressurized samples relating to the untreated control was calculated using the following formula: $RCN = 2^{-\Delta\Delta Ct}$, where ΔΔCt = ΔCt (pressurized) − ΔCt (untreated).

2.5. β-Galactosidase specific activity measurement

Yeast protein extracts and the assay of β-galactosidase activity were performed as described previously [19]. Specific activity was expressed in nanomoles of ONPG (*o*-nitrophenyl-β-D-galactoside) hydrolyzed per minute and mg of protein.

3. Results and discussion

3.1. The double deleted msn2msn4 yeast cells are more sensitive to hydrostatic pressure

The role of the Msn2/4 transcription factors in the cell survival after hydrostatic pressure treatments was evaluated (Fig. 1). When pressurized for 30 min, the double deleted strain Wmsn2msn4 revealed to be slightly more sensitive than the wild-type W303-1A (WT) (Fig. 1a). The difference in cell viability was detected for pressures above 100 MPa, and became more pronounced after 1 h of pressure treatment as shown in Fig. 1b. This result demonstrates that the lack of Msn2/4 reduce cell viability under severe stress conditions agreeing to the survival pattern observed for this strain under high temperature and oxidative stress [22,23].

The pressure sensitivity exhibited by the double mutant Wmsn2msn4 was different from phenotype observed for the single mutant in *MSN2* or *MSN4* in a BY4742 background. Those cell types were around 4–5-fold more resistant to a high-pressure treatment of 125 MPa for 1 h when compared to the wild-type [24]. However, due to the partially overlap in Msn2 and Msn4 function, the sensitivity to stress conditions is usually exhibited by the double mutant, and not by the strains with a single disruption in either *MSN2* or *MSN4* genes [17].

3.2. Msn2/4 are required for high pressure resistance conferred by pressure preconditioning

We have investigated the role of Msn2/4 in the acquired pressure tolerance induced by a previous treatment with a non-lethal pressure of 50 MPa. As seen in Fig. 2, the protection induced by the 50 MPa treatment (PT) could be clearly observed for the wild-type strain W303-1A. The survival rate after the subsequent severe stress of 200 MPa for 30 min (high pressure, HP) was substantially enhanced (10-fold to 10^3-fold for pre-treatments of 30 min and 1 h, respectively) in comparison to the sample that was not previously treated. By contrast,

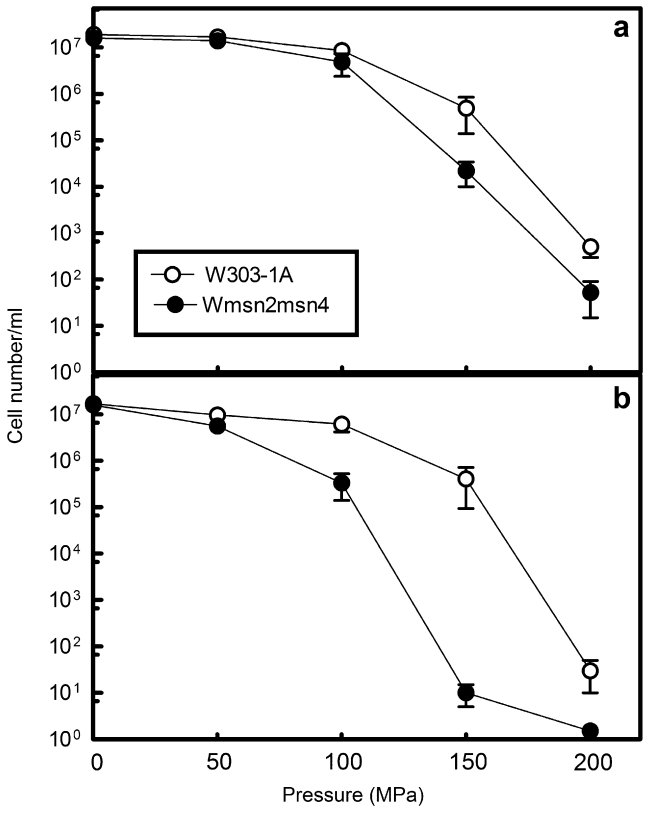

Fig. 1. The double deletion *msn2*Δ *msn*4Δ turns *Saccharomyces cerevisiae* cells more sensitive to hydrostatic pressure. Exponentially growing W303-1A and Wmns2msn4 cells $OD_{600\ nm}$ = 1.0 were subjected to different values of pressure. Cell viability was determined by CFU counting after 30 min (a) or 1 h (b) of pressure treatment. Error bars represent the S.D. of three independent results.

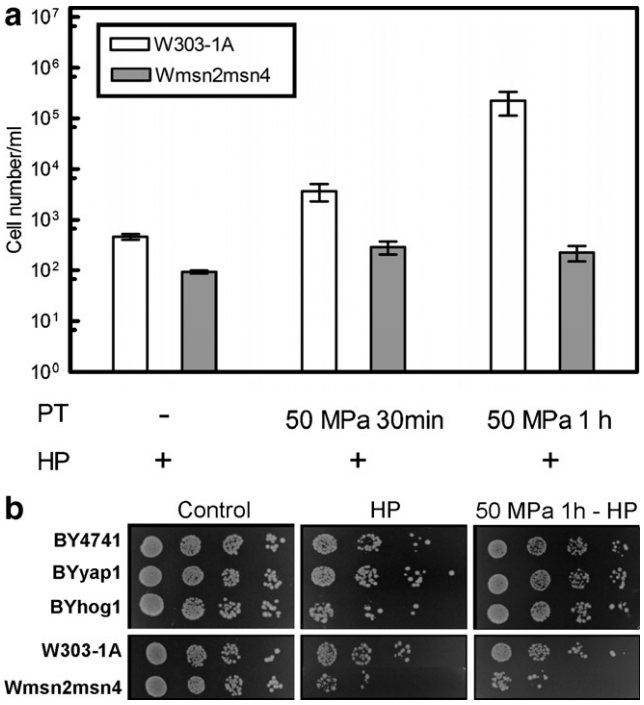

Fig. 2. The acquisition of pressure resistance is impaired in *MSN2* and *MSN4* double mutant (a). Strains W303-1A and Wmns2msn4 at initial concentration of 10^7 cells ml^{-1} ($OD_{600\ nm}$ = 1.0) were assayed for viability after different pressure treatments. Cells were directly subjected at 200 MPa for 30 min (HP), or were pre-conditioned at 50 MPa (PT), incubated 15 min at 1 atm, and then, subjected to HP. BYhog1 or BYyap1 mutants can be pressure pre-conditioned (b). Cells at initial concentration of 10^7 cells ml^{-1} ($OD_{600\ nm}$ = 1.0) were diluted 10-fold and then serially diluted (5-fold each step). The samples subjected to HP or pressure pre-conditioned were directly serially diluted and 6 μl were patched in YPD plates. Plates were incubated at 30 °C for 2 days and analyzed. All experiments were made in triplicate and error bars represent the S.D. of independent results.

the preconditioning treatment of 50 MPa had no protective effect against the 200 MPa pressure stress on the double mutant strain Wmsn2msn4 (Fig. 2a). We also tested the mutants *BYyap1*Δ *and BYhog1* Δ, which are defective in the stress response pathway triggered by high oxidative and high osmotic conditions, respectively (Fig. 2b). The phenotype of the *yap1* Δ mutant was similar to the BY4741 wild-type when tested for pressure preconditioning efficiency. The same result was observed for the *hog1*Δ mutant, though this strain seemed to be slightly more sensitive to the 200 MPa treatment (HP). Therefore, we can conclude that the transcriptional response triggered by Msn2/4 is required for the induction of pressure resistance. This feature is different from other stress conditions such as osmotic, oxidative or heat stress, in which yeast cells can be preconditioned despite the absence of Msn2/4 [17,22,23].

One of the important elements for cell survival to stress conditions is the accumulation of trehalose, which is known to be controlled not only by the activation of *TPS1* trough Msn2/4 transcription factors, but also by change in enzymatic activity [23,25]. In fact, even though tps1Δ mutants are more sensitive to high pressure than the wild-type [20], wild-type yeast cells seem not to accumulate trehalose after a high pressure treatment [26]. Therefore, our data suggest that other components of the stress response controlled by Msn2/4 can be involved in the induction of cell resistance after a pressure preconditioning treatment.

3.3. Hydrostatic pressure activates STRE dependent expression

To test if hydrostatic pressure activates gene expression through Msn2/4 pathway, the gene activation mediated by the stress-responsive *cis*-element STRE was assayed using the strain OL556-STRE carrying a genome integrated *lac*Z reporter gene under the control of four STRE motifs. Exponentially growing cells were subjected to different ranges of hydrostatic pressure for 30 min or 1 h, and β-galactosidase accumulation was measured by specific activity assay (Fig. 3a). β-Galactosidase specific activity was enhanced by pressure treatments of 30 min and the highest induction levels were recorded after 1 h.

Since the STRE dependent gene expression is strongly inhibited by the cAMP-PKA pathway [19], we tested if artificially high levels of cAMP could abolish the STRE dependent induction of β-galactosidase accumulation promoted by a 50 MPa hydrostatic pressure treatment. The OL556-STRE strain is defective in the high affinity phosphodiesterase encoded by the *PDE2* gene. This characteristic, first described as *rca1* mutation, allows the increase in the intracellular level of cAMP by supplementing the medium with this compound, thus preventing Msn2/4 activation [27,28]. As shown in Fig. 3b the β-galactosidase specific activity was increased 12-fold after pressure treatment of 50 MPa for 1 h in the absence of cAMP. By contrast, in the presence of 3 mM cAMP, the specific activity remained very low showing that the STRE dependent

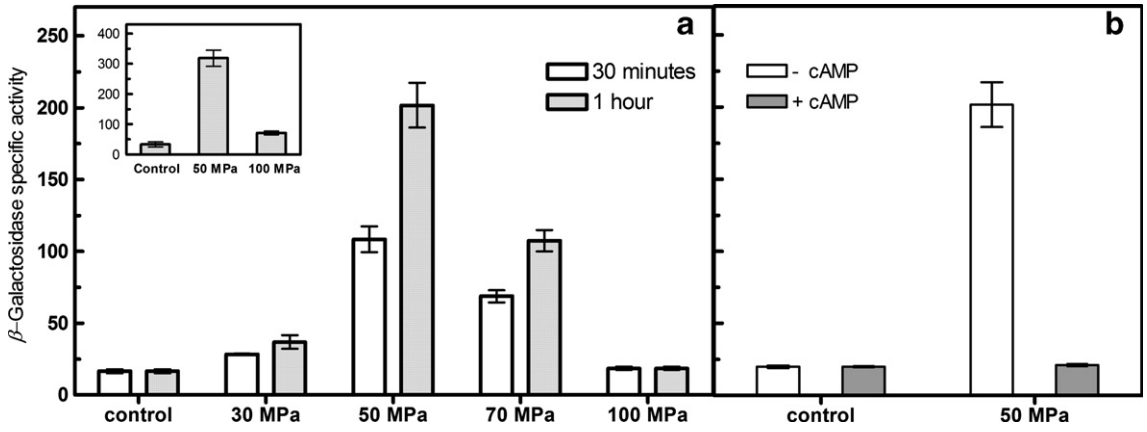

Fig. 3. The intensity of pressure treatments affects the expression level of STRE-*lacZ* reporter gene (a). Strain OL556-STRE was grown in YPD at 27 °C up to $OD_{600\ nm}$ = 1.0 and subjected to pressure treatments for 30 min or 1 h. Cultures were further incubated for 15 min at 27 °C and β-galactosidase specific activity was assayed in crude cell extracts. Inset: The same experimental conditions were performed using the strain W303-1A-STRE. The values corresponding to 1 h treatment after 50 or 100 MPa are demonstrated. Pressure induction of *LacZ* reporter gene mediated by STRE is inhibited by cAMP (b). cAMP (3 mM) was added to the half of the exponentially growing strain OL556-STRE at 27 °C. After 1 h the two cultures were subjected to 50 MPa for 1 h. Control remained at 27 °C. Samples were collected 15 min past the end of the stress treatments and β-galactosidase specific activity was determined. Error bars represent the S.D. of three independent experiments.

induction of gene expression by hydrostatic pressure is negatively controlled by the signaling cAMP-PKA signalling pathway. This evidence is further supported by the observation that cells incubated with 3 mM cAMP were more sensitive to pressure treatment of 200 MPa for 30 min and could not be preconditioned by the mild pressure treatment of 50 MPa for 1 h (Supplementary Material).

By using the STRE-*lacZ* reporter gene, we also observed that the STRE dependent expression varied with the intensity of the applied pressure. After 1 h at 30 MPa a slight increase of β-galactosidase specific activity was recorded, achieving the highest level at 50 MPa (Fig. 3a). However, this activity was weaker following 70 MPa and barely detectable after 100 MPa treatment. To exclude the possibility that this induction profile was due to a strain specific factor, the experiments performed with OL556-STRE was repeated using STRE-LacZ construct in a W303-1A background. Despite the higher activation levels obtained with W303-1A-STRE, the relative response observed among the pressure treatments was in agreement with the ones recorded for OL556-STRE (Fig. 3a-inset). A Wmsn2msn4-STRE construct was also assayed, and as expected, any increase in β-galactosidase specific activity was recorded. Therefore we can conclude that the activation observed in Fig. 3a was specifically induced by Msn2/4 factors.

Furthermore, we checked if the lack of increase in β-galactosidase specific activity after 100 MPa was a consequence of enzyme inactivation due to high pressure: firstly, *lac*Z gene was induced by a heat treatment of 37 °C for 1 h at 1 atm and subsequently transferred to 100 MPa at 25 °C for 30 min. After this period, the pressurized sample still presented most of the activity shown by the unpressurized control (data not shown), demonstrating that β-galactosidase is stable under 100 MPa. Therefore, the lack of STRE dependent *lac*Z induction at 100 MPa suggests either that protein synthesis is impaired at 100 MPa, or that the Msn2/4 transcription factors may be weakly activated at this high pressure value (see below).

These results are in agreement with studies focusing on the optimal pressure pre-treatment able to confer high pressure tolerance in *S. cerevisiae* [26,6]. Both works show that the highest protection level is achieved by a 50 MPa treatment and that 100 MPa preconditioning is unable to confer resistance against high pressure [26,6]. Moreover, cell tolerance is further enhanced if samples are incubated for 15 min at 1 atm before the severe treatment [6].

3.4. Different transcription factors acting at 50 MPa and 100 MPa hydrostatic pressure treatments

To confirm that Msn2/4 signaling pathway contributes to gene expression after a hydrostatic pressure stress, we followed the mRNA expression of *HSP12* in W303-1A and Wmsn2msn4 strain. *HSP12* is a well-known target for Msn2/4 [15,16] and its pressure induction has been detected by different groups [3–5].

The *HSP12* mRNA level was measured by semi-quantitative RT-PCR (Fig. 4a) and Real-time PCR analysis (Fig. 4b). In the wild-type strain, *HSP12* was induced after a 50 MPa and 100 MPa pressure treatments and also by heat shock. In contrast, no induction was observed in the double mutant Wmsn2msn4 after a 50 MPa pressure treatment (Fig. 4). Thus, taking into account this result and the STRE dependent *lacZ* induction (Fig. 3a), it can be concluded that Msn2/4 transcription factors are the main activator of *HSP12* expression in this pressure condition.

When high pressure of 100 MPa was applied, the *HSP12* expression was reduced in the double mutant strain Wmsn2msn4 compared to the W303-1A (Fig. 4b) suggesting a slight contribution of Msn2/4 that could not be detected by β-galactosidase specific activity experiments. However, a 5-fold *HSP12* induction was observed in relation to the basal expression at 1 atm, indicating that other transcription factor besides Msn2/4 may also be implicated in *HSP12* induction at 100 MPa. This induction was accompanied by protein translation as observed by Western-blot (data not shown). The action of transcription factors depending on the stress severity has previously been reported for *HSP12* and *TPS1* induction by heat shock. Msn2/4 dependent transcription is mostly activated at 37 °C while at 40 °C treatment the Hsf1 factor is mainly responsible for gene activation (Fig. 4a) [29,25].

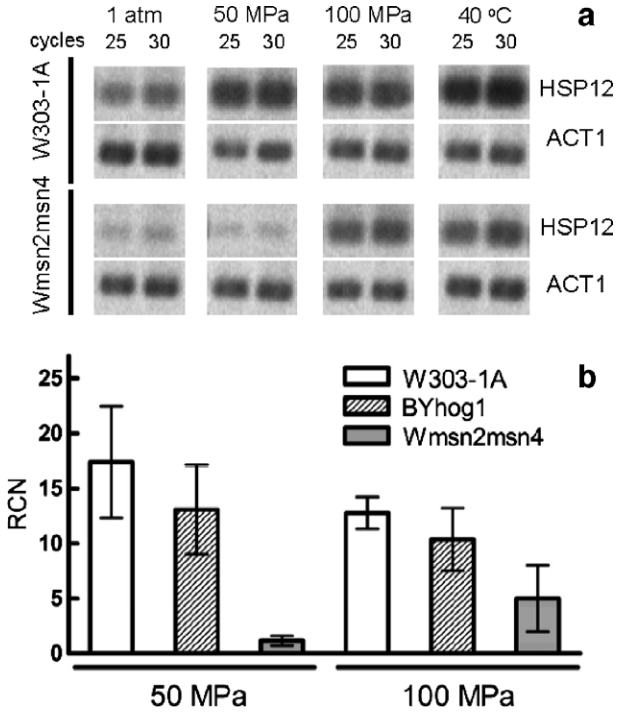

Fig. 4. Msn2/4 transcription factors are required for the induction of *HSP12* gene by hydrostatic pressure. Cells exponentially grown in YPD at 27 °C were pressurized at 50 MPa or 100 MPa for 30 min or shifted at 40 °C for 1 h. After 15 min of incubation at 1 atm, cells were withdrawn for total RNA extraction and RT reaction. Total amount of *HSP12* and *ACT1* mRNA were measured by conventional semi-quantitative RT-PCR analysis (a). Images of the agarose gel show the specific band corresponding to the *HSP12* and *ACT1* amplicons obtained at cycles 25 and 30 of the amplification curve. Concentration of *HSP12* amplicon relative to the control sample of each strain was determined by Real time-PCR analysis (b). The induction values were estimated by the $2^{-\Delta\Delta Ct}$ method where RCN is the relative copy number of *HSP12* first normalized by the internal control (*ACT1*) and then, by the *HSP12* relative expression of the respective untreated controls. Error bars represent the S.D. of three independent experiments.

The Hog1p MAP kinase pathway is activated by high osmolarity stress and is known to activate Msn2/4 [28]. Therefore, we checked if the pressure stress signal is dependent on this pathway. In agreement with the survival data shown in Fig. 2b, the absence of Hog1 did not impair *HSP12* induction (Fig. 4b), although the expression levels were slightly lower than observed at W303-1A. This result suggests that Hog kinase pathway is not essential for Msn2/4 dependent induction of *HSP12* by hydrostatic pressure stress.

This study revealed that high pressure elicits a genuine regulated response in yeast cells, in which Msn2/4 figures as one of the most important factors acting at mild pressures around 50 MPa. The pathways involved in Msn2/4 activation by pressure, may be the same as the ones already proposed to other stresses, such as the Hsp70 recruitment by misfolded proteins leading to inactivation of Ras protein, thus reducing cAMP levels [30] as suggested by Palhano et al. [6]. Alternatively, a direct down regulation of adenyl-cyclase by hydrostatic pressure, as already reported for rat brain membranes, might be responsible for Msn2/4 transcriptional activation [31]. Furthermore, exploring how yeast cells deal with different ranges of pressure may bring new insights to the understanding and identification of new stress controlled pathways.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2006.10.007.

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